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An experimental study to evaluate pathogenicity of *Yersinia ruckeri* isolated from rainbow trout (*Oncorhynchus mykiss*) in Caspian trout (*Salmo caspius*)

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Abstract

The main objective of this study was to evaluate the pathogenicity of isolated *Yersinia ruckeri* from Caspian trout. *Y. ruckeri* specimens were isolated from infected rainbow trout farms located in north of Iran. The identification was confirmed by biochemical tests and 16s rRNA gene sequencing. The pathogenicity test was carried out to determine the virulence of the *Y. ruckeri* by IP injection, and histopathological and hemato-biochemical changes were evaluated pre and post challenge. Based on the results, LD_{50} were calculated as 1×10^5 CFU ml^{-1}, while 1×10^7 CFU ml^{-1} caused 100% mortality after 10 days in the experimental groups. The main histopathological changes were seen in liver, kidney, spleen and intestine, including erosion and necrosis, infiltration of inflammatory cell, hyperplasia and *catarrhal enteritis* in the infected organs. Also, a significant decrease in glucose, RBC counts, Hb values and HCT percentage and significant increase in the WBC counts, neutrophils percentage, AST, ALP and LDH values were observed in infected fish after challenging. Finally, Caspian trout is susceptible to Yersiniosis and can play important role in transmission of *Y. ruckeri* to cultured and wild fish.

Keywords: Caspian trout, *Yersinia ruckeri*, Pathogenicity, Hemato-biochemical changes, Histopathological changes

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Introduction

Enteric redmouth disease (ERM) or Yersiniosis is an important disease that causes economical loss in the aquaculture industry. Yersiniosis is the second most infectious bacterial disease in the coldwater fish farms in Iran; 15 epidemic outbreaks of this bacterial disease were reported in provinces of Iran (Zorriehzahra *et al*., 2012).

EMS as a globally distributed disease is reported in various freshwater and sea water fish. More than twenty species mainly of Salmonide origin are affected so far and this number is likely to increase in the future following the introduction of new species and increased aquaculture trade (Chettri *et al*., 2013). The disease can affect fish of all age classes but is most acute in small fish up to fingerling size. The clinical sings of Yersiniosis depend on species, age and temperature; furthermore, acute and sub-acute forms, characterized by slightly different symptoms and mortality rates, are also known. The most clinical signs of the disease include lethargic behavior and inactivity, swimming near the surface, anorexia, darkening of the skin. Hemorrhages are found on the external surface, at the gill tips, the base of the fins and around the mouth cavity, eyes and lateral line (Danley *et al*., 1999).

The bacterial etiology has been confirmed following the isolation and identification of *Yersinia ruckeri* as a Gram-negative rod with rounded ends of 0.5-0.8×1.0-3.0 μm in size (Tobback *et al*., 2007). This bacteria does not form endospores, a capsule is not present, but often has flagella and strains show variable motility. So far, five O-serotypes, five outer membrane protein (OMP) types and two biotypes of *Y. ruckeri* were recognized (Tobback *et al*., 2007; Wheeler *et al*., 2009).

Caspian trout (*Salmo caspius*, Salmonidae) is one of the most popular fish in Iran due to its economic value (Quillet *et al*., 1992). Caspian trout is native to the western and southern coast of Caspian Sea and is commercially cultured in Iran because of its better growth and higher weight gain compare to other brown trout (Kiabi *et al*., 1999). In few studies, pathogenicity of *Y. ruckeri* were evaluated in salmonid fish such as rainbow trout (Soltani and Tarahomi, 2002; Avci and Birincioğlu, 2005) and brown trout, *Salmo trutta* (Hietala *et al*., 1995). Despite the importance of Caspian trout in the aquaculture industry, there is no reliable database on the pathogenicity of *Y. ruckeri* in this valuable fish. Thus, this study was done to evaluate the pathogenicity of *Y. ruckeri* on Caspian trout via IP injection, as well as to examine histopathological and hemato-biochemical changes pre and post challenge.

Materials and methods

Fish

Two hundred and forty Caspian trout with average weight of 44.15±2.6 g were purchased from Dr Bahonar salmonid governmental farm (Kelardasht, Iran) and transported to

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Sari University of Agricultural Sciences and Natural Resources (Sari, Iran). Fish were acclimatized for 2 weeks in fiberglass tanks (500 L) and fed with a commercial diet (Mazand, Iran) three times a day. The water temperature was maintained at 16.2±1.0°C, dissolved oxygen at 8.3±0.4 mg L^{-1}, pH at 7.74±0.2 and electrical conductivity at 5326.5±236.5 MM cm^{-1}. The photoperiod was set at 14 h light and 10 h dark cycle.

Yersinia ruckeri isolation and biochemical tests

Two hundred moribund juvenile rainbow trout suspected to Yersiniosis were sampled in Mazandaran province farms, north of Iran, during spring 2014 to winter 2014. Suspected fish were transported to the central laboratory of Shahrekord University for bacteriological and molecular examination. Sampling of kidney, spleen and liver was done in the aseptic condition and then were directly streaked by sterile swabs on brain heart infusion (BHI) agar (Oxoid, USA) with adjusted pH of 7.4±0.2. The samples were tested separately for each organ and each fish. Plates were incubated at 25°C for 48h. After macroscopic and microscopic observation of the colonies, single colonies with pure culture growth were subcultured onto BHI agar and identified using conventional biochemical tests recommended by Austin and Austin (2007).

Genotypic characterization of bacterial isolates using PCR

DNA was extracted using a high pure bacterial kit (Mannheim, Germany), according to manufacturer's recommendations. Two pairs of primers including F: (5′- CAG CGG AAA GTA GCT TG–3′) and R: (5′- TGT TCA GTG CTA TTA ACA CTT AA –3) were designed based on the nucleotide sequences of the 16s rRNA gene of *Y. ruckeri* (Akhlaghi and Sharif Yazdi, 2008) and synthesized by CinnaGen company (Tehran, Iran).

The PCR was performed in a total reaction volume of 50 µl containing: 50 mM KCL, 10mM Tris-HCl (pH 9.0), 1.5 mM MgCl, 200 µM dNTPs, 20 pmol of each primer and 2 U *Taq* DNA polymerase per 50 µl reactions and 4µl of template DNA. The PCR reaction was carried out in thermal PCR (Auto-Q server, England) and repeated for 37 cycles: 4 min at 94°C, 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; this was followed by a final extension of 10 min at 72ºC (Akhlaghi and Sharif Yazdi, 2008). The PCR products were separated on 1% agarose gel in 0.5× Tris–borate-ethylene diamine tetra acetic acid (EDTA) buffer and visualized using ethidium bromide and a UV illuminator (EPS-7601, Iran). The resulting DNA sequences were analyzed using nBLAST program (http://www.ncbi.nlm. nih.gov/BLAST) to determine similarity with published sequences.

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Experimental infection

Based on McFarland standard using spectrophotometer (OD=540) three dose of *Y. ruckeri* including: 1×10^5, 10^6 and 1×10^7 CFU ml^{-1} were prepared and the live bacteria count was calculated (Soltani *et al*., 2014). Additionally, one treatment was used as a control group (all treatments were replicated three times). Twelve fiberglass tanks were designed and divided into 4 groups. Twenty fish were kept in each fiberglass tank and challenged by intraperitoneal injection (IP) with 0.1 mL^{-1} suspensions of each concentration of bacteria (Sterile distilled water was injected for the control group). The survival percentage and clinical signs of fish were calculated and recorded every 24 h for 2 weeks (Hietala *et al*., 1995). Also, LD_{50} was calculated using the simplified method that described by Hietala *et al*. (1995).

Blood sampling and hematological analysis

At the first, fish anesthetized with clove oil (50 mg L^{-1}), (Adel *et al*., 2015) then blood samples were collected pre and 10 days post challenge in 1×10^5 CFU ml^{-1} dose (LD_{50} dose) of *Y. ruckeri* from the caudal vein of individual fish. One mL of blood sample (3 pools of five fish) was transferred to a microtube containing EDTA anticoagulant and immediately used for hematological examination, while the 1 ml blood sample was transferred to a non-heparinizes micro tube for determination of biochemical parameters. Sera samples were obtained by blood centrifugation (3000 X g, 15 min) and stored at -80 °C until use.

Red blood cells (RBC: 10^6 mm^{-3}) and white blood cells were enumerated in an improved Neubauer hemocytometer using Hayem's and Turck's diluting fluids (Blaxhall and Daisley, 1973; Jamalzad Fallah *et al*., 2014). Haematocrit (Ht %) was determined by the standard microhematocrit method and expressed as percentage. The haemoglobin (Hb, g dl^{-1}) level was determined according to cyanomethemoglobin procedure. Differential leukocyte cells were carried out by preparing Giemsa stained smears and counted under light microscopy (Adel *et al*., 2015).

Biochemical assay

Alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), total protein (g/dl), glucose levels (mg/dl), albumin (g/dl) were quantified in fish sera using commercial kits (Pars Azmoon, Iran) and a biochemical auto-analyzer instrument (Eurolyser, Belgium), pre and 10 days post challenge in 1×10^5 CFU ml^{-1} dose of *Y. ruckeri* in Caspian trout (Adel *et al*., 2015). Serum globulin (g dl^{-1}) was determined by the following formula: Globulin=total protein − albumin.

Histopathological study

Ten days post challenge in 1×10^5 CFU ml^{-1} dose, the liver, kidney, spleen and intestine of fish were removed by dissection of the abdominal cavity. The

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tissue samples were fixed in 10% buffered formaldehyde solution and followed by alcohol dehydration and embedding in paraffin (Sharifpour *et al*., 2014). Six-micrometer thick sections were stained with haematoxylin-eosin for the study of histomorphometric properties of Caspian trout tissues and were interpreted under a light microscope Model and Country?

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) followed by *t*-tests statistical analyses (*p*-value of<0.05 was considered significant) using SPSS software version 18.

Results

Bacterial isolation and biochemical tests

Bacteriological cultures of the kidney, intestine and liver of rainbow trout showed 38 isolates (38%) of *Y. ruckeri* that isolated from two hundred moribund juvenile rainbow trout specimens. The results of biochemical tests were summarized and compared to descriptions by Austin and Austin (2007) in Table 1.

Table 1: Biochemical characteristics of isolated *Yersinia ruckeri.*

Test	*Y. ruckeri* (our study)	*Y. ruckeri* Austin and Austin (11)
Pigmentation	White	White
Gram	-	-
Motility (12-30 ^{0}C)	+	+
Motility (37 ^{0}C)	-	-
Ornithine decarboxylase	+	+
Production of Indole	-	-
Production of H_2s	-	-
Production of Oxidase	-	-
Production of Catalase	+	+
Nitrate reduction	-	-
Methyl red test	+	+
Voges-Proskauer reaction	-	-
Oxidative-fermentative	F	F
Degradation of gelatin	Variable	+
Degradation of Aesculin	-	-
Production of acid from Maltose	+	+
Production of acid from Sucrose	-	-
Production of acid from Rhamnose	-	-
Production of acid from Inositol	-	-
Production of acid from Mannitol	+	+
Production of acid from Glucose	+	+
Production of acid from Sorbitol	-	-
Growth in: 4-10 °C	+	+
Growth in: 10-37 °C	+	+

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16S rRNA gene sequencing

The PCR assay of 6 *Yersinia ruckeri* isolates gave the expected 409 bp PCR fragment of 16S rDNA sequences (Fig. 1). Homology searches of 16S rRNA gene with nBLAST analysis confirmed as *Y. ruckeri* with 99 to 98% similarities with the published sequences in GenBank.

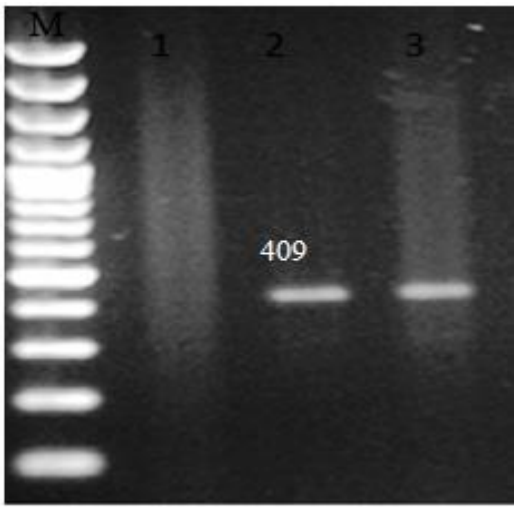


Figure 1: Electrophoretic analysis results (1% agarose gel) of DNA amplified fragments from *Yersinia ruckeri* isolates in this experiment. Lane 1: negative control (distilled water); Lane 2: positive control (KC291153); Lane 3 the isolated bacteria (409 bp). Lane M: Marker 100bp (Fermentas).

Pathogenicity

The first signs of disease and the first mortality in challenged fish observed 48 and 72 hrs after IP injection respectively. Cumulative mortality rate (%) of IP injected Caspian trout with various concentrations of *Y. ruckeri* is shown in Fig. 2. Based on the results, increasing concentrations of bacteria increased mortality rate and clinical signs appeared in a shorter time. A mortality of 50% (LD_{50}) recorded in the fish that were injected with 0.1 mL PBS containing 1×10^5 CFU ml^{-1} *Y. ruckeri* and 100% mortality was observed in *S. caspius* injected with 1×10^7 CFU ml^{-1} of *Y. ruckeri* concentration.

After challenge, tissue samples of kidney, liver and spleen from each dead fish were taken aseptically and cultured on brain heart infusion agar medium and incubated for 48 h at 25°C. Isolated bacterial colonies were then identified based on routine microbiological methods (morphology, biochemical and carbohydrate fermentation tests).

The first clinical signs were lethargy, anorexia and erratic swimming. The main clinical signs that observed post challenge include uni or bilateral exophthalmia, darkness of the skin, abdominal distension, hemorrhages and petechia around the mouth cavity and eyes, basal fin (Fig. 3), skin, gills and in internal organs such as kidney, liver and spleen. No mortality or clinical sign observed in the control group.

Haematological and biochemical analyses

Changes in the haematological and biochemical values of Caspian trout before and after challenge with *Y. ruckeri* are shown in Tables 2 and 3. Based on the results, glucose level between pre and post challenge of bacteria differed significantly ($p< 0.05$) but there was no significant difference between total protein, albumin and globulin pre and post-challenge.

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Figure 3: Hemorrhage and petechia around the mouth cavity and basal fin of Caspian trout after challenged with10^5 CFU ml^{-1} of *Y. ruckeri* 10 days post challenge.

Table 2: Biochemical values of Caspian trout pre and 10 days post challenge with 1×10^5 CFU ml^{-1} of *Y. ruckeri*, letters (e.g. a, b) are used to highlight significant difference.

Parameter	Pre-challenge	Post-challenge	*P* value
ALT (U L^{-1})	18.52 ± 2.3^a	20.60 ± 2.7^a	0.128
AST (U L^{-1})	231.6 ± 24.8^a	289.4 ± 37.3^b	0.002
ALP (U L^{-1})	732.4 ± 38.5^a	842.5 ± 52.3^b	0.006
LDH (U L^{-1})	1232.5 ± 224.2^a	1429.2 ± 326.7^b	0.023
Glucose (mg dl^{-1})	78.49 ± 5.3^a	64.72 ± 6.5^b	0.012
Total PR (g dl^{-1})	2.72 ± 0.18^a	2.48 ± 0.27^a	0.124
Albumin (g dl^{-1})	1.48 ± 0.12^a	1.24 ± 0.10^a	0.325
Globulin (g dl^{-1})	1.22 ± 0.09^a	1.18 ± 0.07^a	0.246

Table 3: Hematological values of Caspian trout pre and 10 days post challenge with 10^5 CFU ml^{-1} of *Y. ruckeri*, letters (e.g. a, b) are used to highlight significant difference.

Parameter	Pre-challenge	Post-challenge	*P* value
RBC ($\times 10^6$ cell ml^{-1})	1.19 ± 0.07^a	0.94 ± 0.03^b	0.005
WBC ($\times 10^3$ cell ml^{-1})	18.4 ± 1.5^a	20.8 ± 1.8^b	0.002
Neutrophils (%)	24.9 ± 0.9^a	28.4 ± 1.3^b	0.021
Lymphocytes (%)	72.1 ± 1.0^a	70.8 ± 0.9^a	0.114
Monocytes (%)	2.1 ± 0.2^a	2.3 ± 0.3^a	0.246
Hb (g dl^{-1})	9.8 ± 0.9^a	8.3 ± 0.6^b	0.026
HCT (%)	32.2 ± 0.6^a	30.9 ± 0.4^b	0.034

Levels of AST and LDH increased significantly ($p<0.05$) post challenge of *Y. ruckeri*; although ALT level in post-challenge increased, the change was not significant ($p>0.05$). The results of WBC and RBC counts, Hb and HCT levels and percentage of neutrophils in post- challenge were significantly less than pre-challenge ($p<0.05$). There was no significant difference between percentage of lymphocytes and monocytes of pre and post-challenge with *Y. ruckeri* in Caspian trout ($p>0.05$).

Histological examination of the tissues

The histopathological damages were observed in several organs of the challenged fish such as liver, kidney, intestine and spleen. Histopathological

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changes of challenged Caspian trout in liver include focal necrosis of hepatocyte, pyknotic nuclei and karyolysis of hepatocytes, mild fat accumulation, hyperemia, fatty changes, sinusoidal dilatation and congestion (Figs. 4 and 5). The histopathological findings in kidney include glomerular vacuolation, increase in the space between glomerulus and Bowman's capsule, the pyknotic nucleus of kidney cells, inflammatory cell infiltration and shortening or destruction of epithelial urinary tubes (Fig. 6). Histopathological changes in intestine include catarrhal enteritis, increase in the goblet and inflammatory cells (lymphocyte, macrophage and plasma cells) infiltration in intestine, destruction in top of the intestinal villi and intestinal epithelium (Fig. 7), accumulation of mucus, colonies of bacteria and necrotic cells in the intestinal lumen. In spleen due to abnormal iron metabolism hemosiderosis in red pulps was observed in 10 days post challenge Caspian trout (Fig. 8).

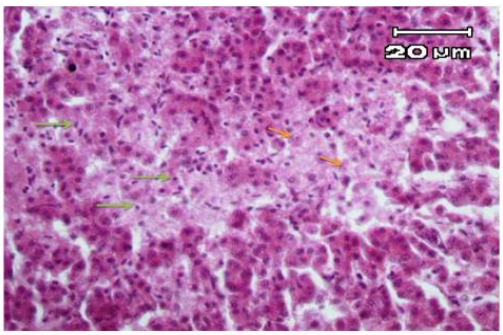


Figure 4: Focal necrosis of liver (green arrows show pyknotic nuclei, orange arrows show karyolysis of liver cells) (H&E. ×40).

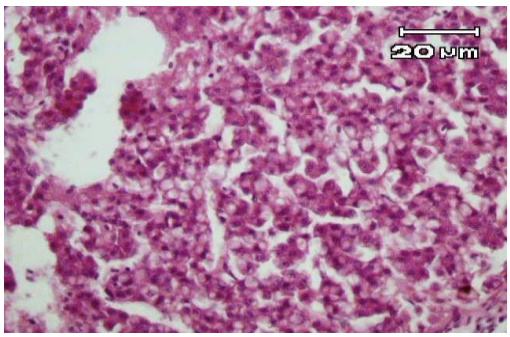


Figure 5: Fatty change and sinus dilation of the liver of Caspian trout challenged with *Y. ruckeri* (H&E. ×40).

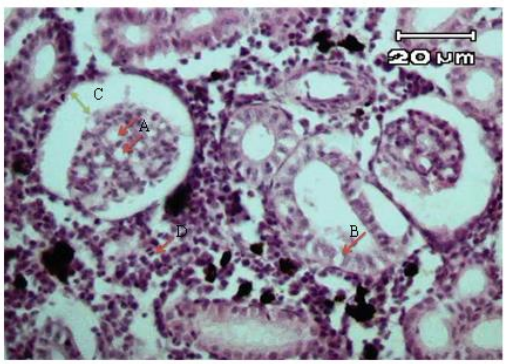


Figure 6: Glomerular vacuolation (A), epithelial cell of urinary duct (B), increased space between glomerulus and Bowman's capsule (C), and pyknotic nucleus (D) (H&E. ×40).

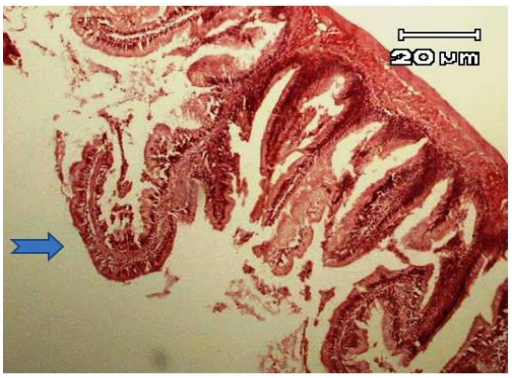


Figure 7: Catarrhal enteritis (blue arrow) in intestine tissue (H&E. ×40).

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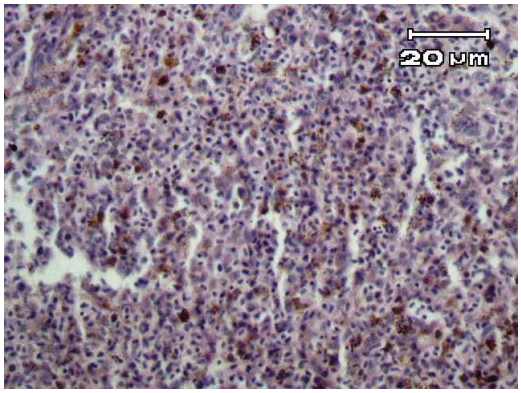


Figure 8: Hemosiderosis in spleen's red pulp of Caspian trout challenged with *Y. ruckeri* (H&E. ×10).

Discussion

In the present study, 38 *Y. ruckeri* strains were isolated from juvenile rainbow trout suspected to Yersiniosis during spring to winter 2014. Biochemical tests such as glucose-fermentative, oxidase-negative and nitrate reduction negative and 16S rRNA gene sequencing positively identified the isolates as *Y. ruckeri*. However, biochemical tests lead to a false negative diagnosis of *Y. ruckeri* infection in channel catfish (Danley *et al*., 1999). As stated by Santos *et al*. (1993), the profile using API 20 E System does not discriminate between *Y. ruckeri* and *Hofniya alvei*, and can misidentify isolates of *Y. ruckeri* as *H. alvei*.

In this study, an identification of Yersiniosis is based on clinical signs and the bacterial pathogen was isolated from systemic sites such as head kidney or spleen. Based on a review by Tobback *et al*. (2007), strains of *Y. ruckeri* can also be classified based on biotype (their capability to ferment sorbitol), serotype and outer membrane protein type. According to Austin and Austin (2007), experimental challenges are not accurate to estimate the pathogenicity level of bacteria due to the procedure that does not mimic natural environment, although they do give some indication of pathogenicity progress. The results obtained in this study showed that initial mortalities in the infected fish were seen first in 72 hrs at 16.2±1.0°C post IP injection. The first signs in fish behavior were noticed including inappetence, swimming near the surface, and moving sluggishly. The present study showed LD_{50} was 1×10^5 CFU ml^{-1}, while 100% mortality caused by 1×10^7 CFU ml^{-1} dose after 10 days. Danley *et al*. (1999) reported IP injected catfish fingerlings with the bacterial isolate at 7.8×10^6 CFU ml^{-1} showed some clinical differences with experimentally infected rainbow trout. In this study, the observed clinical signs were unilateral or bilateral exophthalmia, hemorrhages and red spots around the mouth cavity and eyes, skin darkness, and abdominal distension. However, in immersion experiments of Hietala *et al*. (1995), 50% of fish become *Y. ruckeri* carriers with no sign of ERM disease and mortality was observed after 18 days post infection.

Hematological estimation is proved useful in detection of anemia, total leukocyte and changing leukocyte counts as an indication of the physiological status of fish which provides valuable evidence on the species specific response or range and nature of the pathological status (Rehulka, 2002; Bektas and Ayik,

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2009). Present findings indicated reduced RBC and WBC levels in Caspian trout infected with *Y. ruckeri*. In eel (*Aguilla anguilla*), Yildiz *et al*. (2005) stated the reduction of hematocrit level when infected with *Aeromonas hydrophila*. Altun and Diler (1999) reported the effects of *Y. ruckeri* infection on hematological parameters of rainbow trout. They detected microcytic, normochromic anemia on the third day of experimental infection, and macrocytic, normochromic anemia at days 13 and 15. In the present study, the Hb and HCT contents of fish were lower in post infection that is mainly attributed to the damage of *hematopoietic* organs such as kidney and spleen. A similar findings were obtained after the challenge of *Y. ruckeri* (Soltani *et al*., 2014) and *Streptococcus faecium* in juvenile rainbow trout (Pourgholam *et al*., 2010).

Biochemical analyses showed increasing activity of AST, ALP and LDH values in infected fish after challenge. In a similar study, after experimental *A. hydrophila* infection in Indian major carp (*Labeo rohita*), increase in the levels of AST and ALT was reported (Kumar *et al*., 2006). Liver enzymes are considered as indicators of hepatic activity, and are affected by water quality parameters, the condition of fish cultivation, diseases, food and sex of fish (Shahsavani *et al*., 2010). The elevated levels of AST, ALP and LDH in the current study may be related to the histopathological changes and hepatopathy in infected fish after challenge (Adel *et al*., 2015).

The histopathological results obtained in this study suggested that the pathological findings in several organs of challenged fish such as liver, kidney, intestine and spleen are in agreement with the findings of Avci and Birincioğlu (2005). They concluded that the most pathological findings that could be used to identify rainbow trout infected with *Y. ruckeri* were edema of the gills, focal necrosis of the spleen, mononuclear cell proliferation in kidney and also fatty changes in the liver. However, they found no visible histopathological change in the tubules of the kidney. Danley *et al*. (1999) and Rigos and Stevenson (2001) reported vascular changes in the gills, including hyperemia and hemorrhages in experimentally infected rainbow trout with the bacterium, *Y. ruckeri*. In our study, histopathological findings in intestine were catarrhal enteritis, increase in the goblet and inflammatory cells, and also destruction of top of the intestinal villi and intestinal epithelium. Mahjoor and Akhlaghi (2012) observed histopathological changes including hemorrhages and hyperemia in the tunica muscularis and serosal surface and presence of epithelial cells, RBC and bacteria in lumen in the intestine of naturally infected rainbow trout with Yersiniosis.

This is the first pathogenicity and histological finding of *Y. ruckeri* being evaluated on Caspian trout in Iran. Clearly, additional tests on pathogenicity including the size of fish

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and stress condition factors (such as different water temperatures) should be considered. Information on the pathogenesis in this study will lead to the development of preventive control to efficiently fight this bacterial agent which is urgently needed.

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